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Systemic Macrophage Activation in Locally-induced Experimental Arthritis

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Local and systemic macrophage activation was examined during the course of monoarticular murine antigen-induced arthritis (AIA), induced by systemic immunization and subsequent local induction. The levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12p70, and nitric oxide (NO) were determined in joints, sera, and supernatants of peritoneal macrophages (the latter unstimulated or stimulated *ex vivo* with LPS/IFN-γ). In comparison with normal mice, systemic immunization (day 0) was associated to significant rise of TNF-α in serum, IL-1β in the joints, IL-6 in unstimulated macrophages and IL-12p70 in stimulated macrophages. Local induction led to a further significant increase of: (i) TNF-α, IL-1β, and IL-6 in the joints; and (ii) IL-1β, and IL-6 in sera and stimulated macrophages during acute and/or early chronic AIA (days 1 to 7). Unstimulated macrophages showed increased NO release (day 3), while stimulated macrophages significantly increased secretion of IL-12p70 (day 1). In late chronic AIA (day 21), cytokine/NO expression returned to immunization levels or below at all sites; solely IL-1β in the joints remained significantly above normal levels. Therefore, the prevalently local AIA model is characterized by a mixture of local and systemic activation of the mononuclear phagocyte system (MPS). While systemic MPS activation preceding arthritis induction can be attributed to systemic immunization, further systemic activation during arthritis appears an integral pathogenetic component of AIA.

Introduction

Human rheumatoid arthritis (RA) is a chronic systemic disorder of unknown aetiology, characterized by progressive joint destruction. Crucial pathogenic phenomena are joint inflammation, synovial hyperplasia, and abnormal immune responses [1], with infiltration of T-helper (Th) cells and macrophages into synovial tissue (reviewed in [2–4]). Antigen-induced arthritis (AIA), an intensively-studied experimental arthritis model, shows homologies to human RA in terms of histopathology and responses to several immunomodulatory drugs [5]. AIA is induced by systemic immunization with antigen (methylated bovine serum albumin; mBSA) in complete Freund's adjuvant (CFA), followed by single intra-articular injection of the antigen into the knee joint cavity [6].

Several lines of evidence suggest that activated macrophages play a relevant role in arthritis through: (i) processing and presentation of (auto)antigens to T cells; (ii) production of the pro-inflammatory mediators; tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, nitric oxide (NO) and other radicals; and (iii) production of tissue-degrading enzymes (reviewed in [3, 4]). TNF-α and IL-1, for example, may crucially affect mechanisms of joint destruction [7] or reactivate AIA [8]. IL-6 can act synergistically with IL-1 in the acute-phase reaction during inflammation, activating T cells and inducing terminal differentiation of B cells into plasma cells [9]. Activated macrophages also produce IL-12, a cytokine that critically influences the Th1/Th2 balance in favour of a Th1 pro-inflammatory activity (reviewed in [10]) and has a pro-arthritogenic role [11], at least partially in an autocrine fashion.

Finally, upregulation of the inducible isoform of nitric oxide synthase (iNOS), induced in macrophages and synoviocytes by various stimuli [12, 13], results in increased formation of NO; this mediator inhibits the synthesis of proteoglycans and is elevated in the synovial fluid of RA patients [14, 15].

However, the occurrence of systemically-secreted cytokines and their influence on locally-induced arthritis remains to be elucidated. This study focussed on the longitudinal course of systemic and local

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cytokines prevalently produced by macrophages in murine AIA. Although a contribution of other cell types (in particular synovial fibroblasts and chondrocytes) to the cytokine levels cannot be excluded, the expression of these mediators was interpreted as a sign of macrophage activation. The advantage of AIA over other animal models lies in the exactly-defined initiation of arthritis, elicited by antigen injection into the knee joint cavity. The analyses included the acute and chronic stages of disease, as well as the preceding immunization status. Normal mice served as controls. Local macrophage activation was assessed by analysis of the pro-inflammatory mediators TNF-α, IL-1, IL-6, IL-12p70 (the active form of IL-12, a candidate stimulus of macrophage activation), and NO in the inflamed joints, while the serum and peritoneal macrophages harvested from the same animals (both unstimulated and after *ex vivo* stimulation) were analysed for signs of systemic activation.

Materials and Methods

Animals

Female C57BL/6 mice, 7–9 weeks of age, and male/female C3H/J mice, 4–8 weeks of age, were obtained from the Animal Research Facility, Beutenberg Campus, Friedrich Schiller University, Jena, Germany. They were housed under standard conditions, in a 12-h light/dark cycle, and fed with standard pellets (Altromin #1326, Lage, Germany) and water *ad libitum*. All animal studies were approved by the governmental commission for animal protection.

Induction of arthritis

On days −21 and −14, C57BL/6 mice were immunized by subcutaneous injection of 100 µg methylated bovine serum albumin (mBSA) in 50 µl saline, emulsified in 50 µl complete Freund's adjuvant (Sigma, Deisenhofen, Germany; adjusted to a concentration of 2 mg/ml of heat-killed *Mycobacterium tuberculosis*, strain H37RA/ Difco, Detroit, MI, USA). In addition, intraperitoneal injection of 2×10^9 heat-killed *Bordetella pertussis* (Pertussis Reference Center, Krankenhaus Berlin-Friedrichshain, Germany) was performed. Arthritis was elicited on day 0 by sterile injection of 100 µg mBSA in 25 µl saline into the right knee joint cavity, while the left knee joint remained untreated. Animals were analyzed before (non-arthritic mice systemically immunized with mBSA; day 0) and at various time-points after AIA induction (days 1, 3, 7, and 21). Normal mice served as controls. All measurements were performed in at least three independent experimental series, yielding comparable results.

Joint swelling

Mice were anaesthetized before immunization (normal), before arthritis induction (day 0), and on days 1, 3, 7, and 21 of AIA. The knee joint diameter was measured using an Oditest vernier calibre (Kroeplin Längenmesstechnik, Schlüchtern, Germany). Joint swelling was expressed as the difference in diameter (mm) between the right (arthritic) and left (control) knee joint.

Histology and grading of arthritis

After sacrificing the mice, both knee joints were removed *in toto*, skinned, and fixed in 4.5% phosphate-buffered formalin. After decalcification in EDTA, the joints were embedded in paraffin, cut into 5-µm-thick frontal sections, and stained with haematoxylin–eosin or safranin O for microscopic examination. The extent of joint inflammation (as defined by density of leukocytes infiltrated in the synovial membrane/joint space and degree of synovial lining layer hyperplasia) and the degree of joint destruction (cartilage necrosis, bone erosion, and pannus formation) were examined in four sections per knee joint by two independent observers (P.K.P., S.H.) and graded blindly using a semiquantitative score with 0=no, 1=mild, 2=moderate, and 3=severe alterations.

Analysis of serum cytokines

Mice were anaesthetized and killed by cervical dislocation. Sera were collected by total bleeding from the carotid artery and stored at −70°C. Analysis of murine TNF-α, IL-1β, IL-6, and IL-12p70 was performed with commercially available ELISA kits according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). The sensitivity of the assays was 5 pg/ml for murine TNF-α, 3 pg/ml for IL-1β or IL-6, and 2.5 pg/ml for IL-12p70.

Cytokine analysis in joint extracts

Whole joints were taken at various time points of AIA development, snap-frozen in isopropane/liquid nitrogen, and stored at −70°C until further analysis. Joint extracts were obtained according to Smith-Oliver *et al.* [16], i.e., the frozen joints were ground under liquid nitrogen with a mortar and pestle. The powdered tissue was transferred to a glass homogenizer, and exactly 2 ml of sterile saline were added. The powder suspension was homogenized by hand for 2 min and centrifuged for 20 min at 1500×*g* and 4°C. The supernatant was spun again at 300×*g* for 10 min, and the resulting supernatant aliquoted and frozen at −70°C. The assays proceeded using commercially available ELISA kits for murine TNF-α, IL-1β, IL-6, and IL-12p70 (R&D Systems; see above).

Isolation and stimulation of peritoneal macrophages

Peritoneal cells were harvested by peritoneal lavage of six to eight individual C57BL/6 mice with 7 ml cold phosphate-buffered saline (PBS) containing 5 IU/ml heparin (Liquemin N 2000, Hoffmann-La Roche, Grenzach-Whylen, Germany), before and at various times after arthritis induction. The washed cells were resuspended at a density of 1×10^6/ml in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% foetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Jenapharm, Jena, Germany), 100 µg/ml streptomycin (Grünenthal, Stolberg, Germany), 0.5 µM 2-mercaptoethanol (Gibco), and 10 mM Hepes (Gibco; hereafter complete RPMI 1640 medium). A total of 1×10^6 cells/ml/well were seeded in 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany), and allowed to adhere for 2 h at 37°C and 5% CO_2. After attachment, non-adherent cells were removed by washing with warm medium and recounted to calculate the number of adherent cells in the well by subtraction. The remaining adherent cells consisted of >95% macrophages [17]. Following incubation for 24 h with or without the combination of 1 µg/ml LPS (*E. coli*, serotype O26:B6; Sigma) and 1 ng/ml recombinant murine IFN-γ (R&D Systems), the cell culture supernatants were removed, frozen in aliquots, and stored at −70°C until analysis of cytokines by bioassays (TNF-α, IL-1, and IL-6) or ELISA (IL-12p70), as well as NO analysis by the Griess reaction. For TNF-α determination, the cells were cultured in serum-free macrophage medium (Macrophage-SFM, Gibco). LPS-contamination of all media and solutions was <1 ng/ml (LAL-test; E-Toxate, Sigma).

TNF bioassay

TNF activity in culture supernatant of peritoneal macrophages was determined by a cytotoxicity bioassay, using the L929 fibroblast cell line [18]. Briefly, 5×10^4 L929 cells were plated in 96-well flat-bottom plates (Greiner Bio-One), duplicates incubated for 20 h with serial dilutions of test supernatant or recombinant murine (rm) TNF-α (R&D Systems), followed by viability assessment with crystal violet staining. The TNF-α concentration in the test samples was calculated by comparing the dilutions of rmTNF-α and the test samples inducing 50% cytolysis. Assay sensitivity was determined as 2 pg/ml murine TNF-α. Assay specificity was demonstrated by complete blocking of rmTNF-α activity (5 pg/ml) by addition of 100 pg/ml anti-TNF mAb (clone V1q).

IL-1 bioassay

IL-1 activity in culture supernatants was determined by a bioassay using co-stimulation of murine thymocytes [19]. Briefly, thymocytes from C3H/J mice were suspended at a density of 1×10^7/ml in complete RPMI 1640 medium. Duplicates (1×10^6 cells/well) were cultured in 96-well flat-bottom plates (Greiner Bio-One) for 70 h at 37°C and 5% CO_2, in the presence of 1 µg/ml concanavalin A (ConA; Pharmingen, Heidelberg, Germany) and either serially diluted (2-fold) macrophage supernatant or rmIL-1β (R&D Systems). ^{3}H-thymidine (0.5 µCi; specific activity 29 Ci/mmol; Amersham Buchler, Braunschweig, Germany) was added to each well for the last 22 h of incubation. The cells were harvested and the thymidine incorporation was measured. The IL-1 concentration in test samples was calculated by comparing the dilutions of rmIL-1β and the test samples inducing a 4-fold increase of thymidine incorporation. Assay sensitivity was 10 pg/ml IL-1β. In this bioassay, both IL-1α and IL-1β are biologically active, therefore the test sample concentrations were specified as IL-1.

IL-6 bioassay

IL-6 activity in culture supernatants was determined using the IL-6-dependent B9 cell line as described previously [20]. Briefly, 2.5×10^3 B9 cells (duplicates) were plated in 96-well plates (Greiner Bio-One), then serial dilutions of test supernatant or rmIL-6 standard (R&D Systems) were added and the cells incubated for 72 h at 37°C. For the last 4 h, 50 µg (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) were added to each well. Cells were then lysed with 10% SDS in 50% dimethylformamide and the absorbance read at 570 nm. The IL-6 concentration in test samples was calculated by comparing the dilutions of rmIL-6 and the test samples inducing half-maximal proliferation of B9 cells. Sensitivity of the assay was determined as 100 pg/ml rmIL-6. The specificity was validated by showing that neutralizing antibodies abrogated all effects of rmIL-6.

Measurement of NO production

The concentration of nitrite, which is proportional to NO production, NOS activity [21], and NOS-mRNA expression [22], was determined as described previously [20]. Briefly, test samples (100 µl) were mixed with an equal volume of Griess reagent in a 96-well microtitre plate (Greiner Bio-One). Absorbance was read at 570 nm and the test sample nitrite concentrations were calculated using known concentrations of sodium nitrite ($NaNO_2$) as a standard. Assay sensitivity was 1 µmol/l $NaNO_2$.

Statistical analysis

The multi-group Kruskall Wallis test was used for statistical evaluation of the data. Only those parameters that revealed significant differences ($P\leq0.05$) were further analysed with the non-parametric Mann–Whitney *U*-test. A *P*-value of ≤0.05 was considered to be statistically significant. Analyses

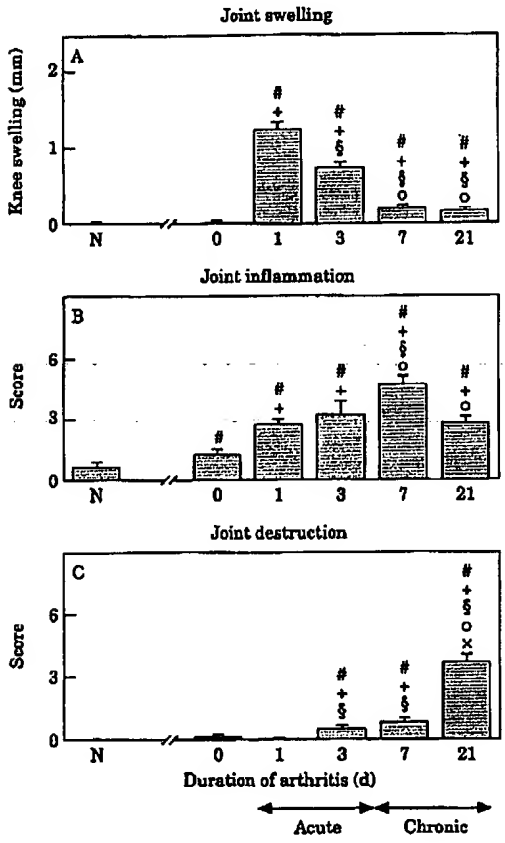

Figure 1. Time-course of AIA (days 0, 1, 3, 7, and 21) as measured by joint swelling (A) and histological scores of joint inflammation (B) and joint destruction (C) (see methods for details). Results are expressed as means±SEM of at least six individual animals per group. $^{\#}P \leq 0.05$, compared to normal control animals (N); $^{+}P \leq 0.05$ compared to immunized, non-arthritic mice (day 0), $^{\S}P \leq 0.05$ compared to day 1, $^{o}P \leq 0.05$ compared to day 3, $^{x}P \leq 0.05$ compared to day 7 of arthritis.

were performed using the SPSS 10.0® program (SPSS Inc., Chicago, IL, USA).

Results

Clinical disease activity

Joint swelling reached its maximum on day 1 of arthritis, and gradually declined until day 7 (Figure 1A). During the acute phase (days 1 and 3), joint swelling was significantly elevated compared to the chronic phase (days 7 and 21). In the chronic phase, the joint diameter remained significantly elevated in comparison with normal control mice and with mice which had only been systematically immunized (day 0; Figure 1A).

During arthritis, the joint inflammation score, as defined by hyperplasia of the synovial lining layer and cellular infiltration (Figure 1B), reached its maximum on day 7 and declined thereafter. In contrast, the joint destruction score, as defined by pannus formation, as well as cartilage and bone erosion, became significantly elevated on day 3 but reached its maximum only during late chronic arthritis (day 21; Figure 1C). Thus, the following phases could be identified: immunization (day 0), acute phase (days 1 to 3), early chronic (day 7) and late chronic phase (day 21).

Cytokine levels in joint extracts

In the immunization phase (day 0), therefore even before the injection of antigen into the joints, there was already a significant elevation of IL-1β compared to normal joints (Figure 2B). These, in turn, contained only trace amounts of IL-1β.

During the acute phase (days 1 and 3), the joint extracts displayed significant elevations of TNF-α, IL-1β, and IL-6 in comparison to the immunization phase (Figure 2A, B & C). On day 7, TNF-α and IL-6 returned to immunization levels (Figure 2A & C). IL-1β also significantly declined compared to day 3, however it remained significantly elevated compared with normal controls (Figure 2B). IL-1β remained significantly above normal controls also on day 21, i.e., well into the late chronic phase of AIA. In terms of degree of cytokine expression, the elevations of IL-1β and IL-6 were of the same order of magnitude (Figure 2B & C), reaching levels of approximately 1 ng/ml. In comparison, the elevation of TNF-α was moderate (Figure 2A), reaching only approximately 35 pg/ml.

Cytokine levels in the supernatants of AIA peritoneal macrophages

Unstimulated macrophages derived from the peritoneal cavity of AIA mice spontaneously released large quantities of IL-6 (approximately 20–30 ng/ml; Figure 2F), which significantly differed from those in normal mice throughout the course of AIA. The levels measured in samples obtained during AIA, however, were not significantly different from those observed upon systemic immunization (day 0; Figure 2F). Significant differences were instead observed between the acute (days 1 and 3) and late chronic phase of AIA (day 21; Figure 2F). There was no spontaneous release of TNF-α or IL-1 (Figure 2D & E).

Upon *ex vivo* stimulation with LPS *plus* IFN-γ, in contrast, AIA peritoneal macrophages produced significant levels of TNF-α and IL-1 (Figure 2G & H), i.e., significantly more than stimulated peritoneal macrophages derived from normal control mice (TNF-α, IL-1) or immunized mice (IL-1). Stimulated AIA peritoneal macrophages also significantly increased their production of IL-6 (Figure 2I). While in the case of TNF-α there was a single significant peak on day 1

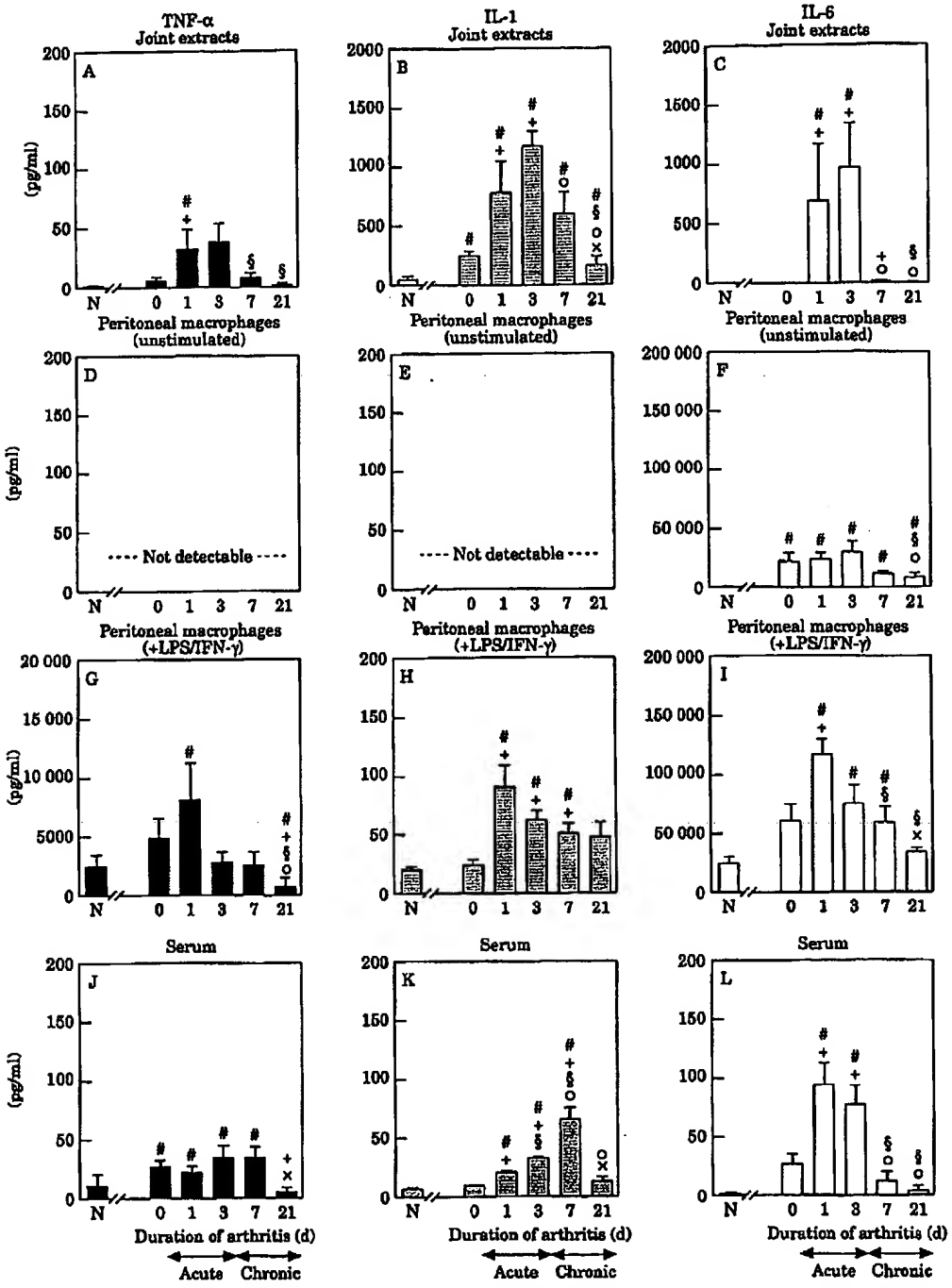

Figure 2. Cytokine concentrations (TNF-α, IL-1, and IL-6) in different compartments (joint extracts, A–C; supernatants of unstimulated and *ex vivo* stimulated peritoneal macrophages, D–I; and serum, J–L) during the course of AIA (days 0, 1, 3, 7, and 21) was measured by ELISA (joint extracts, sera) or bioassay (supernatants of macrophage cultures). Results are expressed as means±SEM of at least six individual animals per group. $^{\#}P \leq 0.05$, compared to normal control animals (N); $^{+}P \leq 0.05$ compared to immunized, non-arthritic mice (day 0), $^{\S}P \leq 0.05$ compared to day 1, $^{\circ}P \leq 0.05$ compared to day 3, $^{\times}P \leq 0.05$ compared to day 7 of arthritis.

(Figure 2G), in the case of IL-1 and IL-6 the significant elevations extended to days 1, 3 and 7 (Figure 2H & I), matching the course of acute and early chronic disease (Figure 1A).

Cytokine levels in serum

The immunization status (day 0) was characterized by significant elevation of serum TNF-α (Figure 2J). A slight elevation of IL-6, in turn, was not significant compared to normal controls (Figure 2L). In the acute phase of arthritis, there was a significant elevation of TNF-α, IL-1β, and IL-6. For TNF-α, the elevations did not exceed those observed in immunized animals (day 0), maintaining a plateau between day 1 and day 7, and then dropping to normal control levels on day 21 (Figure 2J). Notably, the TNF-α elevation in the serum was of the same order of magnitude as that observed in joint extracts (Figure 2A), only more prolonged during the course of AIA (until day 7) and already visible upon immunization (day 0). In the case of IL-1β, the profile of the cytokine mount (significantly above normal and immunized mice) was opposite to that of the joint swelling (Figure 1A), as IL-1β slowly rose to reach a late peak only on day 7 (Figure 2K). On day 21, the IL-1β levels decreased significantly to normal control levels (Figure 2K). In the case of IL-6, significant peaks above normal and immunized mice were reached on day 1 and 3 of AIA. These declined significantly to normal levels on day 7 (Figure 2L).

IL-12p70

In the joints, minute amounts of IL-12p70 (<5 pg/ml) were detectable already in normal animals. These levels did not change upon immunization (day 0), but showed a minor, significant peak above normal levels during early acute AIA (day 1), with a significant decrease until day 7 (Figure 3A).

While resting peritoneal macrophages did not release any IL-12p70 (Figure 3B), *ex vivo* stimulated cells released considerable amounts of this cytokine (max. 250 pg/ml) (Figure 3C). The elevation was significant already upon immunization (day 0) in comparison with normal controls, but became more prominent on days 1 and 7 of AIA. Between these two time points, IL-12p70 dropped significantly on day 3 ($P<0.0001$; Figure 3C). This apparently biphasic course matched the peak of joint swelling on day 1 and appeared to accompany the transition into early chronicity (day 7; Figure 1A). This biphasic course was confirmed in three independent experimental series (data not shown).

Minute levels of IL-12p70 (max. 5 pg/ml) could also be detected in the serum of AIA mice, however they only minimally and non-significantly exceeded those of normal or immunized mice (Figure 3D).

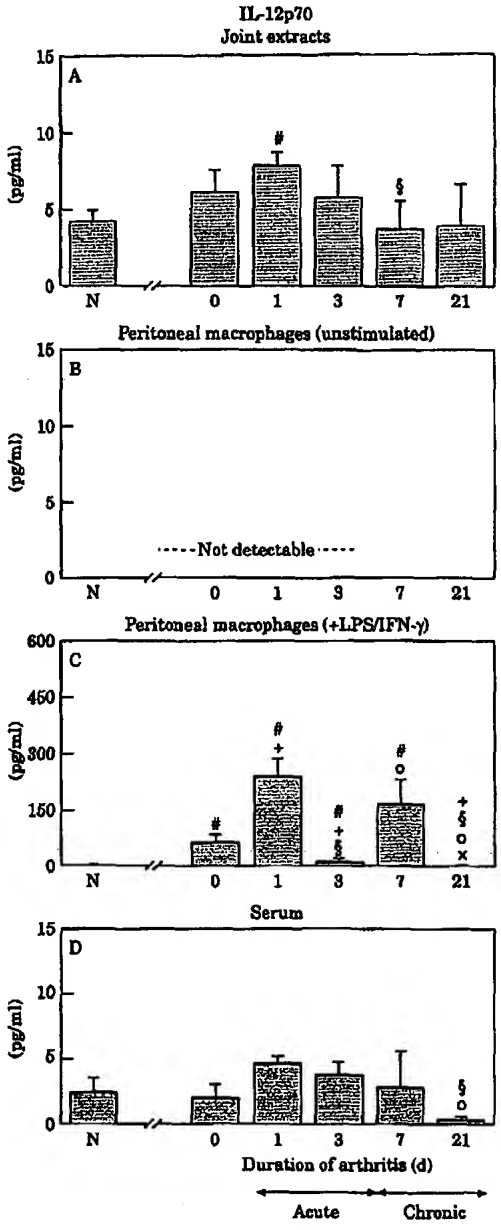

Figure 3. Concentration of IL-12p70 in different compartments (joint extracts, A; supernatants of unstimulated and *ex vivo* stimulated peritoneal macrophages, B–C; and serum, D) during the course of AIA (days 0, 1, 3, 7, and 21), as analysed by ELISA. Results are expressed as means±SEM of at least six individual animals per group. #$P\leq0.05$, compared to normal control animals (N); +$P\leq0.05$ compared to immunized, non-arthritic mice (day 0), §$P\leq0.05$ compared to day 1, °$P\leq0.05$ compared to day 3, ×$P\leq0.05$ compared to day 7 of arthritis.

Nitric oxide

NO was detectable only in the supernatants of AIA peritoneal macrophages. In unstimulated macrophages, the mean NO levels showed a significant peak above normal and immunized mice on day 3 (Figure 4B), while stimulated peritoneal macrophages only demonstrated a minor (1.5-fold), but significant peak above normal levels on day 1 of AIA, with a significant decrease thereafter (Figure 4C). The negative results in joint extracts (Figure 4A) and serum (Figure 4D) were not explainable by the presence of inhibitors of the Griess reaction in the respective compartments, since the addition of joint extracts or serum samples to known NO standards did not influence the expected results (data not shown).

Discussion

Cytokine profile during the immunization status

The immunization status immediately preceding the induction of local AIA (day 0) was characterized by signs of systemic macrophage activation, i.e., elevation of TNF-α in the serum and considerable release of IL-6 and IL-12p70 by peritoneal macrophages. The systemic involvement of macrophages is well-compatible with an MPS response to the spread of antigen and/or adjuvant from the injection site, in addition to a response to intraperitoneally-injected adjuvant (*Bordetella pertussis*).

Interestingly, at this preclinical stage cytokine production was also evident locally in the joints, as shown by significant elevations of IL-1β in comparison to normal. This partial macrophage activation at the local site is probably the result of repeated leakage of systemically-applied antigen into the joint [23], and is supported by local detection of IL-6 and IL-10 at this stage of murine AIA [24].

The source of serum TNF-α remains unclear. Because the serum levels at this stage clearly exceed the joint levels, a spill-over from the joints is highly unlikely. Previous studies in the prearthritis phase of other experimental arthritides [25, 26] map significant TNF-α and IL-1β mRNA production to the draining lymph nodes (but, for example, not the spleen), suggesting lymph nodes as a source of serum TNF-α. Whether elevated TNF-α production by activated circulating monocytes contributes to increased TNF-α levels in serum, in analogy to human rheumatoid arthritis [27], remains a matter of further investigation.

Cytokine profile during acute AIA

Acute AIA (days 1 and 3) was characterized by a significant rise of TNF-α, IL-1β, and IL-6 in the joints, as well as IL-1β and IL-6 in the serum, thus with a more complex cytokine pattern compared to the immunization phase.

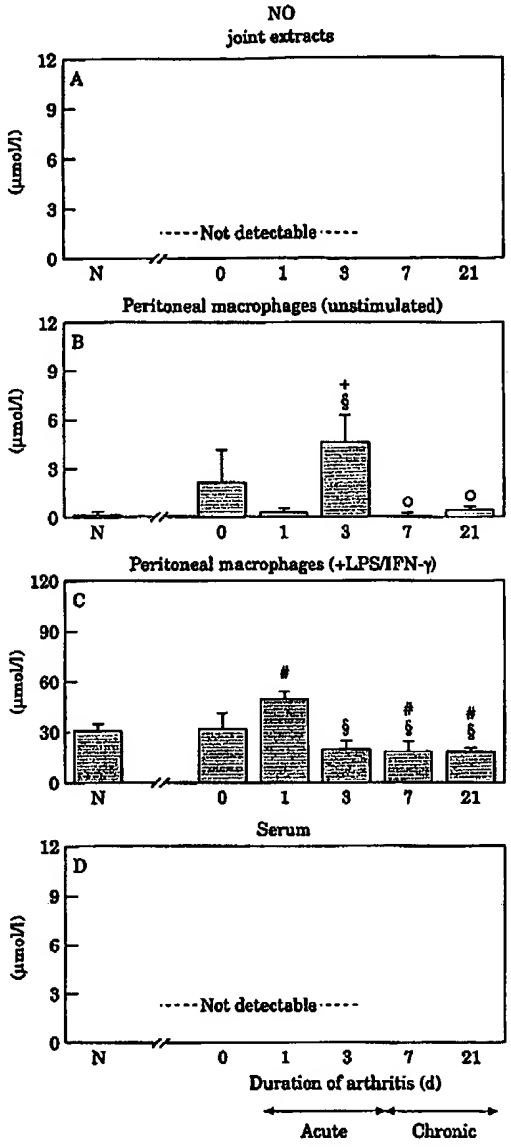

Figure 4. Nitrite concentration in different compartments (joint extracts, A; supernatants of unstimulated and *ex vivo* stimulated peritoneal macrophages, B–C; and serum, D) during the course of AIA (days 0, 1, 3, 7, and 21) was measured using the Griess reaction. Results are expressed as means±SEM of at least six individual animals per group. #$P \leq 0.05$, compared to normal control animals (N); *$P \leq 0.05$ compared to immunized, non-arthritic mice (day 0), §$P \leq 0.05$ compared to day 1, °$P \leq 0.05$ compared to day 3 of arthritis.

In the joint, the cytokine elevation matched the course of acute disease. However, the cytokine peak showed a 2-day delay in comparison with the peak of the joint swelling. These findings are well-consistent with the dynamics of the cellular infiltration, especially of macrophages [28], and stress the

importance of macrophage-derived cytokines in acute arthritis [24]. Quantitatively, TNF-α levels in the joints were approximately 25–40 times lower than those of IL-1β and IL-6. These findings stress the critical role of IL-1β and IL-6 in acute arthritis [3], and agree with the knowledge that in this animal model counteraction of IL-1β is more effective than counteraction of TNF-α [29]. On the other hand, the present data do not exclude that low TNF-α levels may be sufficient to influence the acute phase of experimental and human arthritis [25, 26, 30].

Presence and magnitude of IL-12p70 in joint extracts of AIA show good correspondence with data in experimental collagen-induced arthritis [31] and human RA [32, 33], and support a pathogenetic role for even small amounts of this cytokine also in AIA. Notably, the significant peak of IL-12p70 was reached on day 1, i.e. 2 days before the peak of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. This time course is compatible with a primary macrophage-activating role of IL-12 in AIA [11].

The lack of NO in the AIA joints, on the other hand, represents a clear difference in comparison with human RA [4] and experimental arthritides, such as AIA in rabbits [34] and rats [20]. Since the presence of inhibitors of the NO detection assay was excluded, the present findings probably reflect species differences in the AIA model.

In the serum, TNF-α elevation lasted until day 7, however at a constant level from the immunization phase. This stable course does not reflect the minor increase of this cytokine in the joints, and therefore suggests alternative production sites. At the equivalent stage of rat adjuvant arthritis, for example, TNF-α mRNA is expressed almost exclusively in the spleen [26]. IL-1β and IL-6, in contrast, showed serum peaks that generally matched the course of joint swelling, suggesting a possible spill-over from the joints. However, in the case of IL-1β, the peak levels were reached on day 7, i.e., 6 days after the joint swelling peak.

Whilst clear elevations of serum IL-6 have been previously observed in human RA [35, 36] and experimental arthritides including AIA [20, 37–39], elevated levels of IL-1 protein have been described in adjuvant arthritis [40], and, at least in some studies, in human RA [41, 42], but not in AIA [39]. Although the role of systemic elevation of IL-1β and IL-6 in AIA is presently unclear, evidence for a systemic pro-inflammatory influence of IL-6 on AIA has recently been reported in IL-6 knock-out mice [43].

Unstimulated peritoneal macrophages from AIA mice produced large amounts of IL-6 and low, but detectable amounts of NO, probably as a reaction to bacterial adjuvant and/or systemic spreading of antigen. In addition, peak production of IL-6 in unstimulated peritoneal macrophages, as well as TNF-α, IL-1, IL-6, and NO in LPS/IFN-γ-stimulated peritoneal macrophages, temporally coincided with or even preceded the time-course of swelling and cytokine production in AIA joints. This suggests parallel activation of macrophages in the joint and the peritoneal cavity during AIA, as previously observed in other experimental arthritis models [4]. Interestingly, IL-12p70 elevation in stimulated peritoneal macrophages was biphasic (days 1 and 7, with a significant drop on day 3), stressing that peritoneal macrophages may participate in the dual role of IL-12 during arthritis, i.e., disease promotion in early disease, but disease suppression in late phases [44].

Cytokine profile during chronic AIA

IL-1β was the only cytokine to remain significantly elevated in the joints during chronic AIA, similarly to findings in other arthritis models [40, 45]. The temporal coincidence with the progression of joint destruction, starting in the early chronic phase (see Figure 1C), is compatible with a central importance of this cytokine for the destruction of cartilage and bone in experimental arthritis [46, 47]. Parallel, selective elevation of IL-1β in joints, macrophage supernatants, and serum (although at clearly reduced levels compared to the acute phase), suggests that macrophage may primarily use IL-1β as a mediator for their functions in chronic disease [26]. This is in clear contrast to the simultaneous elevation of all investigated cytokines in the acute phase of AIA.

General considerations

In spite of its local character, AIA exhibits clear systemic signs of macrophage activation, i.e., the elevation in the serum of the pro-arthritogenic cytokines TNF-α and IL-1β, as well as the increased levels of IL-6, a cytokine involved in the acute phase response circuit. Also, peritoneal macrophages show signs of activation in AIA, both constitutively and upon additional *ex vivo* stimulation. Since the systemic signs appearing in acute AIA mostly exceed those observed upon systemic immunization, systemic MPS activation appears to be an integral pathogenetic feature of this local arthritis model, presumably driven by leakage of antigen and pro-inflammatory stimuli from the inflamed joints. This systemic feature of arthritis can clearly be observed also in human RA [27, 48, 49], and makes systemic counteraction of MPS activation an attractive target for therapeutic intervention [50, 51].

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